INNOGENETICS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

SMITH et al.		Atty. Ref.:	**2551-49**
Serial No.	**09/662,462**	Group:	**1634**
Filed:	**September 15, 2000**	Examiner:	**Goldberg**

For: **NUCLEIC ACID PROBES AND METHODS FOR DETECTING CLINICALLY IMPORTANT FUNGAL PATHOGENS**

* * * * * * * * * * * *

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION

I, Geert JANNES declare that:

1. I am a citizen and resident of Belgium and reside at E. Vanhoorenbekelaan 23/1, 3010 Leuven, Belgium.

2. I hold a MSc. degree in Biotechnology, obtained from the State University of Ghent, Belgium in 1988. Specializing in nucleic acid diagnostics since 1991, I am the head of the R&D Theranostics Microbiology Program at Innogenetics, the assignee of the above-identified application. I became Senior Scientist in Innogenetics' Research organization in 1999. I am a co-inventor of the invention claimed in the above-identified patent application.

3. I have read and understood the Office Action dated October 8, 2002 in the above-identified application and the Interview Summary of March 13, 2003 stating that "A comparison with the closest prior art is required". I have read the specification and pending claims of the above-identified application.

Smith et al.
Serial No. 09/662,462

4. I have been advised that the Examiner stated during the interview that the most relevant probes of the cited art are Bothelo's (Yeast, Vol 10, pgs 709-717, 1994) ITS1 *Candida albicans* probe (i.e., ANAB1 - CTTTGAAACAAACTTGCTTTGGCGGTGGCCCAGCCTG), and Lott's (U.S. patent No. 6,242,178) *Candida dubliniensis* ITS2 sequence (i.e., SEQ ID NO:35 - AAGGCGGTCTCTGGCGTCGCCC).

5. I have also been advised that the Examiner may find the presently claimed invention patentable over the cited art if it were demonstrated that these probes of the cited art (i.e. ANAB1 of Bothelo and SEQ ID NO:35 of Lott) are not able to perform (i.e. distinguish *Candida albicans* and *Candida dubliniensis*, respectively) under the same hybridization conditions as the probes of the claims.

6. It is my belief that one of ordinary skill in the art will appreciate that hybridization characteristics of a probe may be significantly affected by, for example, a modification of the length, a shift of one base pair therein, the inclusion of one mismatch, possibly combined with elongation of one or more nucleotides, etc. These modifications would be expected to result generally in modification of hybridization conditions. Contrary to this expectation of one of ordinary skill in the art, the following demonstrates that, as described in the above-identified application, the probes of the presently claimed invention hybridize under the same hybridization and wash conditions and allow simultaneous hybridization to ITS nucleic acids.

7. I have performed, or had performed under my direction, the following experiments in order to demonstrate that the two above-noted probes of the prior art are not able to act as probes under the same hybridization conditions. This performance sharply contrasts with the probes and probe combinations of the presently claimed invention, which have been specifically designed with the purpose of performing under the same hybridization conditions.

8. **DNA extraction**. The following protocol was applied for each of the two samples, one containing *Candida albicans* (strain NCPF 3302 obtained from the National Collection of Pathogenic Fungi, Mycology Reference Laboratory, PHLS Central Public Laboratory, London) and the other containing *Candida dubliniensis* (strain CD36, supplied by Derek Sullivan, Moyne Institute of Preventative Medicine, Trinity College, Dublin, Ireland).

25 ml of yeast culture was harvested by centrifugation at 6,000 rpm (5,000 x g, Beckman J2-21 centrifuge) for 15 min and washed once in distilled water. The cell pellet was resuspended in 7 ml of lysis buffer (10 mM Tris-HCl [pH 8.0], 250 mM EDTA [pH 8.0], 0.5% Triton X-100 [vol/vol]) supplemented with 3 mg of lyticase enzyme (Sigma-Aldrich Ltd.) per ml and incubated at 37°C overnight. Spheroplasts were subsequently lysed by incubating the samples with 3 mg of proteinase K (Roche Diagnostics, Mannheim, Germany) per ml at 55°C for 2 h. The proteinase K was inactivated at 95°C for 10 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; Sigma-Aldrich) was added, the tube was mixed by inversion and centrifuged at 12,000 rpm (19,800 x g; Beckman J2-21 centrifuge) for 1 h. The aqueous layer was transferred to a fresh tube, and an equal volume of ice-cold isopropanol was added. The DNA was centrifuged at 12,000 rpm for 15 min. The pellet was washed twice with 70% ethanol. Air-dried pellets were resuspended in 200 µl of 0.1 x TE buffer (Sigma-Aldrich Ltd.) and stored at –20°C.

9. **PCR amplification.** PCRs were performed in a final volume of 100 µl. PCR conditions for amplification of the ITS region of fungal species, in particular *Candida albicans* and *Candida dubliniensis*, were as follows.

Each reaction mixture contained 0.25 mM deoxynucleotide triphosphates (dU/dNTPs [2:1]), 1× reaction buffer (Promega), 3 mM $MgCl_2$, 1 U of uracil DNA glycosylase (Roche), 2.5 U of Taq polymerase (Promega), , and 40 pmol each of the forward and reverse primers. To amplify the full ITS (ITS1 + ITS2), the primer pair ITS5 / ITS4 was used. And to amplify the ITS1, primer pair ITS5 / ITS2 was used.

Five µl of template DNA was added, and the volume was adjusted to a final volume of 100 µl with nuclease-free water (Sigma-Aldrich, Ltd.).

Cycling conditions for amplification of the ITS were as follows: 37°C for 10 minutes for one cycle, followed by 94°C for 2 min for one cycle, followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and DNA extension at 72°C for 2 min, with a final extension cycle at 72°C for 10 min. A no-template negative control was included in each PCR run.

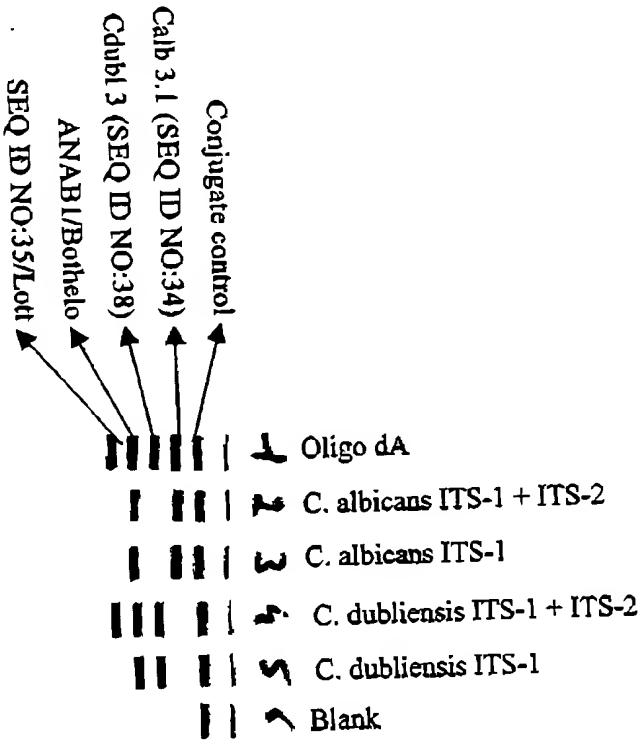
The primers used were those described in the above-identified application (page 15, lines 5-22), they were biotinylated:
ITS2 GCTGCGTTCTTCATCGATGC
ITS4 TCCTCCGCTTATTGATATGC
ITS5 GGAAGTAAAAGTCGTAACAAGG

10. The **INNO-LiPA fungal assay** is based on the reverse-hybridization principle. Oligonucleotide probes for the LiPA were enzymatically provided with a polydeoxythreonine tail. Subsequently, probes were immobilized as parallel lines onto a nitrocellulose membrane, with the top line containing a positive-control biotinylated DNA.

10 µl of biotinylated PCR product was denatured in a LiPA tray by adding an equal volume of denaturing solution (NaOH-EDTA) and incubating at room temperature for 5 min. A 1-ml aliquot of preheated (50°C) hybridization buffer (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS) together with a LiPA strip was added and incubated at 50°C for 30 min in a shaking water bath. The strips were stringently washed three times in 1 ml of hybridization buffer: twice for 1 min at room temperature and once at 50°C for 15 min. These washes were followed by one wash in 1-ml of rinse solution for 1 min followed by incubation in 1 ml of an alkaline phosphatase-linked streptavidin conjugate at room temperature for 30 min. The strips were washed twice in

1 ml of rinse solution and once in 1 ml of substrate buffer for 1 min each at room temperature. Finally the strips were incubated in 1 ml of substrate solution (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium diluted in substrate buffer) for 30 min at room temperature. The color reaction was stopped by adding distilled water to the strips. After drying, the strip results were interpreted by eye.

11. **Interpretation of the strips.** The following Figure shows the results of the hybridization test with LiPA strips.



From the hybridization pattern obtained when using the ITS1 *Candida albicans* ANAB1 of Bothelo and the ITS2 *Candida dubliniensis* SEQ ID NO:35 of Lott probes together, one of ordinary skill would conclude that *Candida dubliniensis* is present in both samples. In contrast to the results obtained with the probes of the cited art, a *Candida albicans* ITS1 probe of the present invention (i.e., SEQ ID NO:34 - GGTTTATCAA CTTGTCACACCAGA - Calb 3.1) and a *Candida dubliniensis* ITS1 probe of the present

invention (i.e., SEQ ID NO:38 - GGTTTTGTTCTGGACAAACTT - Cdub1 3) are able to simultaneously distinguish the *Candida albicans* and *Candida dubliniensis* samples. Consequently, the ANAB1 probe of Bothelo and the SEQ ID NO:35 probe of Lott cannot be used together in the conditions used for the probes of the presently claimed invention for the detection and identification of *Candida albicans* and *Candida dubliniensis*.

12. **Conclusion.** I believe that the experimental data obtained and described above and in the attached demonstrate that the presently claimed invention was not obvious and provides unexpected benefits which include the ability to distinguish the recited organisms under the same hybridization conditions.

The Examiner is urged to appreciate that the presently claimed invention provides more than 40 probes (19 relating to *Candida* species) that can all be used together under the same hybridization conditions. This is a significant advancement in the art. Many obstacles had to be overcome, in particular, for example, the modification of some probes, adding one or more nucleotides, in order to maintain a high level of specificity and sensitivity in, for example, the LiPA assay. The probes of the claimed invention not directly derived from the ITS sequence but, in many cases, include nucleotide(s) that cannot be found in the ITS. This is the case, for example, for the probes of SEQ ID NO:33, 34, 35, 37 and 38 of the present invention.

The probes of the presently claimed invention provide an inventive selection of probes that, theoretically, may have been derived from the ITS sequence known in the prior art. I do not believe however that one of ordinary skill in the art would have been able to predict from the cited art which of these probes would hybridize with a sufficient specificity and sensitivity to be able to detect and identify *Candida albicans*, *Candida dubliniensis*, *Candida kefyr*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis*, as claimed.

13. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

Signed this 7th day of July, 2003.

Geert JANNES